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13. ABSTRACT *(Maximum 200 words)*

The major outcomes of the current work are: (1) Development of a new molecular strategy, mRNA extraction from soil, assesses the catabolic activity of soil bacteria in situ. (2) Quantitative the association between the biosensor bioluminescence response and the PAHs bioavailability present in the waste environment. (3) Demonstration the ability of NAH plasmid to mediate the initial biodegradation reactions in the catabolic pathway of fluorence. The current research work is focuses on developing new molecular diagnostics' method for measuring in situ PAH biodegradation activity and co-related the bioluminescence response, that produced by a naphthalene-lux reporter strain, to the bioavailability of different pollutants in the real environment. In addition, catabolism of a tricyclic aromatic hydrocarbon, fluorene, mediates by a NAH plasmid is also investigated.

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Annual Progress Report (Jan. - Dec. 1993)

Molecular Ecology of Bacterial Population in Environmental Hazardous Chemical Control

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Background

The major outcomes of the current work are:

(1) Development of a new molecular strategy, mRNA extraction from soil, assesses the catabolic activity of soil bacteria *in situ*.

(2) Quantitative the association between the biosensor bioluminescence response and the PAHs bioavailability present in the waste environment.

(3) Demonstration the ability of NAH plasmid to mediate the initial biodegradation reactions in the catabolic pathway of fluorene.

Research Progress

The current research work is focuses on developing new molecular diagnostics' method for measuring *in situ* PAH biodegradation activity and co-related the bioluminescence response, that produced by a naphthalene-lux reporter strain, to the bioavailability of different pollutants in the real environment. In addition, catabolism of a tricyclic aromatic hydrocarbon, fluorene, mediates by a NAH plasmid is also investigated.

Molecular diagnostics of PAH biodegradation in manufactured gas plant soils. Traditional methods for quantifying specific catabolic bacterial populations underestimate the true population count due to the limitations of the necessary laboratory cultivation methods. Likewise, *in situ* activity is also difficult to assess in the laboratory without altering the sample environment. To circumvent these problems and achieve a true *in*

situ bacterial population count and activity measurement, new methods based on molecular biological analysis of bacterial nucleic acids were applied to soils heavily contaminated with polycyclic aromatic hydrocarbons (PAHs). In addition, a naphthalene-lux reporter system was used to determine bioavailability of naphthalene within these soils. DNA extracted from seven PAH-contaminated soils and hybridized with the *nahA* gene probe indicated that the naphthalene degradative genes were present in all samples in the range of 0.06 to 0.95 ng/100 ml DNA extract which was calculated to represent 3.2×10^6 to 1.1×10^{10} cells/g soil (assuming one copy of these genes per cell). ^{14}C-naphthalene mineralization was observed in all contaminated soils with $^{14}CO_2$ mineralization rates ranging from 3.2×10^5 to $304,920.0 \times 10^{-5}$ µg/g soil h. Phenanthrene, anthracene, and benzo[a]pyrene were mineralized also in several soils. Messenger RNA transcripts of *nahA* were isolated and quantified from 4 soils. Only one soil tested, soil B, was inducible with salicylate above the *in situ nahA* gene transcript level. Two of the soils, C and G, were already fully induced *in situ*. The naphthalene mineralization rate correlated positively with the amount of *nahA* gene transcripts present (r=0.99). Naphthalene was bioavailable in soils A, D, E, G, and N as determined by a bioluminescent response from the naphthalene-*lux* reporter system. Taken together, these data provided information on what the naphthalene-degrading bacterial population was experiencing *in situ* and what approaches would be necessary to increase activity.

Environmental application of *nah-lux* reporters. A bioluminescent reporter bacterium *Pseudomonas fluorescens* HK44 for naphthalene and salicylate catabolism was used for analysis of naphthalene and bioavailability in aqueous extracts of soils contaminated with petroleum hydrocarbons. For each experimental set which involved two different soils, one presumably uncontaminated soil A and a heavily contaminated soil B, a series of naphthalene concentrations were included in order to prepare bioluminescence versus naphthalene standard curve. For all the samples investigated, the final biomass concentrations were

identical and remained unaffected by the contaminants. A very reproducible bioluminescence response was obtained from contaminated extracts as compared to extracts from an uncontaminated control soil; however, quantitative estimates of the amount of naphthalene present in the mixture were not accurate. On studies conducted with JP-4 jet fuel experimentally contaminated samples a linear correlation between the relative amount of pollutant and the magnitude of the bioluminescence response was observed. It was interesting to note that 64 mg/l toluene, 22 mg/l p-xylene and 980 mg/l acetone caused a significant bioluminescence increase as compare to a control with water. In addition, the mixtures of the same concentrations of either acetone and toluene or acetone and p-xylene the responses were exactly additive but not for the mixture of toluene and p-xylene. The solvent effects were dependent on the physiological status of the reporter culture and were present in growing, but not in resting reporter cultures.

In order to determine the relationship between bioluminescence response and pollutants, a mRNA extraction was performed on 4 ml culture in the test vials. The analysis of nah-lux mRNA in growing cultures revealed that even though toluene had a strong effect on the bioluminescence response in growing reporter cultures after exposure to the solvent, but it did not increase *nahG-luxCDABE* gene expression. In contrast, nah-lux mRNA levels significantly increased after exposure to naphthalene or JP-4 jet fuel. It is proposed that the increase in bioluminescence after exposure to solvents was due to changed fatty acid synthesis patterns affecting the aldehyde supply for the bioluminescence reaction.

Bioluminescent biosensor for on-line pollutant monitoring. A optical whole cell biosensor based on the bioluminescent catabolic reporter bacterium, *Pseudomonas fluorescens* HK44, was developed for continuous, rapid on-line monitoring of naphthalene and salicylate bioavailablity and microbial catabolic activity in waste stream, i.e. 'dirty' samples. The reporter strain, *P. fluorescens* HK44, carries a *nah-lux* reporter plasmid (pUTK21)

which contains a transcriptional gene fusion between a *luxCDABE* gene cassette from *Vibrio fischeri* and the *nahG* gene of the salicylate operon, and is capable to degrade naphthalene and salicylate. The reporter culture ($O.D._{546}$=0.8) was washed once and resuspended in a sterile 0.9% NaCl solution. The suspension (12.5 ml) was mixed with 2X of a sterile, low viscosity alginate solution 3.5% (w/w) in NaCl solution (0.9%) and with 7.5 ml sterile glycerol. Biosensor probe tips were prepared by injecting the cell-alginate mixture into the ferrule cavity on the liquid light guide. Then, the ferrule was immediately immersed into a stirred $SrCl_2$ solution (0.1 M) in order to harden the strontium-alginate matrix. This biosensor probe was inserted into a measurement cell which received simultaneously the waste stream solution and a maintenance medium.

Under defined conditions, a rapid increase in bioluminescence was detected after exposure to naphthalene and salicylate. The magnitude of the response and the response time were concentration dependent. The response time was defined as the time interval between exposure to the pollutant and the time when the bioluminescence response exceeded the sum of three standard deviations of the average baseline value prior to induction. It was interesting to note that at lower inducing substrate concentrations of 0.5 mg/l salicylate and 1.55 mg/l naphthalene, the response times of 24 minutes were significantly longer than at high concentrations. Under repetitive perturbation conditions, good reproducibilities of the signal magnitude and response time were found for both substrates, naphthalene and salicylate. The specificity of the biosensor was determined by examining the response of different carbon sources in the waste stream and compared to the response to naphthalene. The bioluminescent responses were insignificant (less than 2 fold) and response times were significantly longer than those observed for naphthalene (1.55 and 15.5 mg/l) when glucose (1.0 g/l) and YEPG medium (1.0 g/l) were used as substrate. Exposure to toluene resulted in no significant bioluminescence signal. The environmental application of the biosensor was tested using

real, complex pollutant mixtures containing naphthalene. A bioluminescence response was detected after exposure to an aqueous solution saturated with JP-4 jet fuel. Naphthalene concentration was detected at 0.55 mg/l in the effluent of the biosensor. A positive bioluminescence response was also observed after exposure to the aqueous leachate from a manufactured gas plant (MGP) soil. The concentration of naphthalene was estimated at 0.6 mg/l in the effluent of the soil column.

Biotransformation of fluorene mediated by a NAH plasmid. Fluorene, a tricyclic aromatic hydrocarbons and a typical by-product of coal-conversion which contains a five-member ring, is formed from the combustion of fossil fuels and has been found in vehicle exhaust emission, crude oils, waste incineration and cigarette smoke. It is not a carcinogenic compound, but this compound is highly toxic to fish and aquatic algae. The strain *P. fluorescens* 5RL, contains a bioluminescent reporter plasmid (pUTK21) and exhibits a Nah^+Sal^- phenotype, was used in this study. The primary goal of this study was an evaluation of the ability of NAH plasmid to carry out the degradation of fluorene.

Characterization of metabolites formed from fluorene was carried out by biotransformation experiments. Incubation of the substrate without strain 5RL was used as abiotic control. A yellow color product was observed in the biotransformation study, however, the physical property the compound was not able to determine at present time. Preliminary results obtained from TLC plate suggest that there were accumulation of metabolites in the studies. Identification of metabolites were further analyzed by GC/MS. In neutral extraction, three metabolites were identified by GC/MS by comparison with the authentic standard or mass spectrum obtained from previous published paper. The compound 9-fluorenol (m/z=181) and 9H-fluoren-9-one (m/z=180) were identified with GC retention time of 13.4 and 13.5 minutes, respectively. The third metabolite had a GC retention time of 7.8 minute and was identified as 1-indanone (2,3-dihydro-1H-inden-1-one) with a parent peak (M^+) of m/z=132 and a base peak of m/z=104. To our knowledge, this compound, 1-indanone, was

never reported as an intermediate in the degradation of fluorene by either bacteria or fungi. This result suggested that the NAH plasmid present in strain 5RL has the ability to mediate the degradation of fluorene and the five-member ring is not the initial target for the enzymatic ring-cleavage reaction. The metabolites detected in the acid extraction were not able to identified at present stage.

A bioluminescent assay has been developed to ascertain whether the metabolite(s) produced from biotransformation of fluorene by the *nah* operon capable of interacting with the *nahR* gene product to induce the *nah* and *sal* operons. Bioluminescent assays were performed in triplicate experiments. Fluorene was added to the vials in a solution of DMF to a final concentration of 10 mg/l. Negative controls consisted of DMF only while positive controls contained 10 mg/l of salicylate. Bioluminescent values were obtained after one hour exposure. A T test of the data showed no significant difference between the negative control and the fluorene cultures. While the positive control showed a significant bioluminescence response indicating induction of the *nah* and *sal* operons. These results suggested that fluorene biotransformation does not provide metabolite(s) which can interact with the regulatory protein of the NAH system. It also suggests that the biotransformation of fluorene by NAH plasmid is a cometabolism process.

Publications associated with this investigation.

Atlas, R.M., G. Sayler, R.S. Burlage, and A.K. Bej (1993). Molecular approaches for environmental monitoring of microorganisms. BioTechniques. 12:706-717.

Heitzer, A., B. Applegate, O.F. Webb, T.J. Phelps, G.S. Sayler, D.C. White (1994). Catabolic Bioluminescent Reporter Bacteria: Physiological Aspects of Environmental Applications. Appl. Environ. Microbiol. In review.

Heitzer, A., K. Malachowsky, J.E. Thonnard, P.R. Bienkowski, D.C. White, and G.S. Sayler (1994). Optical Biosensor for Environmental On-Line Monitoring of Naphthalene and Salicylate Bioavailability Using an Immobilized Bioluminescent Catabolic Reporter Bacterium. Appl. Environ. Microbiol. In review.

Heitzer, A. and G.S. Sayler (1993). Monitoring the Efficacy of Bioremediation. Trends in Biotechnology. 8:334-343.

Heitzer, A., O.F. Webb, P.M. DiGrazia and G.S. Sayler (1993). "A Versatile Bioluminescent Reporter System for Organic Pollutant Bioavailability and Biodegradation."In: (R.A Minear and A. Ford, eds.) Molecular Biological Tools in Environmental Chemistry, Biology and Engineering. In press.

Menn, F.-M., B.L. Applegate and G.S. Sayler (1993). NAH plasmid mediated catabolism of anthracene and phenanthrene to naphthoic acids. Appl. Environ. Microbiol. 59:1938-1942.

Nadeau, L., F.-M. Menn, A. Breen and G.S. Sayler (1993). Degradation of DDT by *Alcaligenes eutrophus*. Appl. Environ. Microbiol. 60:51-55.

Sanseverino, J., B.M. Applegate, J.M. Henry King and G.S. Sayler (1993). Plasmid-mediated mineralization of naphthalene, phenanthrene and anthracene. Appl. Environ. Microbiol. 59:1931-1937.

Sanseverino, J., C. Werner, J.T. Fleming, B.M. Applegate, J.M.H. King, and G.S. Sayler (1993). Molecular diagnostics of polycyclic armoatic hydrocarbon biodegradation in manufactured gas plant soils. Biodegradation. In press.

Sayler, G.S (1993). Environmental Biotechnology's Furture in Hazardous Waste Management. In: (W. Tien, L. Lo, S. chen, Y. Shuy, eds.) Biotechnology: Theory and Applications 1993, p.248-254.

Presentations and Abstracts

Applegate, B., L. Lackey, J. McPherson and F.-M. Menn (1993). A bioluminescent reporter for the co-oxidation of trichloroethylene (TCE) by the toluene dioxygenase in *Pseudomonas putida* F1. Abstract Q-108, p365. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Heitzer, A., J.E. Thonnard and G.S. Sayler (1993). Continuous on-line pollutant monitoring using a whole cell biosensor based on a bioluminescent catabolic reporter bacterium. Abstract Q-97, p364. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Menn, F.-M., J. Sanseverino, B. Applegate and G. Sayler (1993). NAH plasmid mediated catabolism of polycyclic aromatic hydrocarbons. Abstract Q-348, p.122. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Nadeau, L., F.-M. Menn, A. Breen and G. Sayler (1993). The aerobic degradation of 1,1,1-trichloro-2,2-bis(4-

chlorophenyl)ethane (DDT) by *Alcaligenes eutrophus*. Abstract Q-158, p.51. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Sanseverino, J., C. Werner, J. Fleming, B.M. Applegate, J.M. Henry King and G.S. Sayler (1993). Molecular diagnostic of polycyclic aromatic hydrocarbon biodegradation in manufactured gas plant soils. Abstract Q-349, p.410. American Society for Microbiology (ASM) Annual Meeting, Atlanta, GA.

Lectures and Seminars (G.S. Sayler, 1993)

American Academy of Microbiology, Strategies and Mechanisms for Field Research in Environmental Remediation, San Antonio, TX. Invited participant and discussion leader.

Oklahoma State University, Department of Microbiology and Cell Biology, "Environmental Molecular Diagnostics", Stillwater, OK. Invited Seminar.

TOCOEN Toxic Organic Compounds in the Environment, "Molecular Technologies Applied to PCB and PAH Biodegradation Analysis", Znojmo, Czech Republic, Invited participant.

University of Washington, Department of Microbiology, "Molecular Approach in Biodegradation Assessment", Seattle, WA. Invited seminar.

NIEHS Biodegradation Workshop, "Molecular Approaches for Biodegradation Assessment", Triangle Park, NC. Invited seminar.

American Society of Microbiology Annual Meeting, "Application of Gene Fusions in Monitoring Gene Expression in Degradative Bacteria *In situ*, Atlanta, GA., Invited speaker.

Celgene Corporation, "Microbial and Molecular Monitoring of Biodegradation Processes", Warren, NJ, Invited seminar.

Keystone Molecular Biology Symposium, Environmental Bioremediation and Biodegradation, "Molecular Strategies in Biodegradation Process Monitoring and Optimization" Tahoe City, CA., Invited participant.

American Chemical Society Southeast Regional Meeting, "Bioluminescent Reporter Technology: Genetic Engineering for Chemical and Microbial Process Sensing" Johnson City, TN Invited Presentation.

Environmental Protection Agency, Frontiers in Bioprocessing III, "Molecular Site Assessment and Process Monitoring in Bioremediation and Natural Attenuation", Boulder, CO, Invited Lecturer.

Chilean Association of Microbiology, Symposium on Biodegradation of Industrial Wastes and Pesticides, "Application of Molecular Biology in Measuring Microbial Biodegradation of Organic Pollutants", Santiago, Chile, Invited Participant.

ONR/NRL Environmental Quality Seminar Series, "Lux-Gene Fusions: Bioluminescent Reports for Environmental Biodegradation and Biosynthesis", Arlington, VA, Invited Lecturer.